Diagnostic, predictive, and prenatal testing for facioscapulohumeral muscular dystrophy: diagnostic approach for sporadic and familial cases

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Abstract

Facioscapulohumeral muscular dystrophy (FSHD) is one of the common inherited neuromuscular disorders. The major gene involved, FSHD1, has been localised to chromosome 4q35. This 4q35 locus, detected by pE13-11 (D4F104S1), shows a mutation frequency of about 10% of the incidence. New mutants are characterised by de novo deletions of tens to hundreds of kilobases of DNA. Although these deletion fragments are very useful as a molecular genetic tool, their use in diagnostic DNA testing is hampered by multiple factors, particularly in familial cases. In this report we describe a protocol that can be used for DNA testing in well defined familial cases or proven de novo cases, and in the differential diagnosis of muscular dystrophy patients clinically suspected of having FSHD. In addition, we describe a prenatal diagnosis performed for FSHD1.

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Key words: facioscapulohumeral muscular dystrophy; DNA diagnosis.

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Facioscapulohumeral muscular dystrophy (FSHD) is a neuromuscular disorder characterised by progressive weakness and atrophy of the facial, shoulder girdle, and upper arm muscles. The manifestation of the disease is quite variable, both within and between families. The disease has its onset usually between the first and second decade, and progresses gradually over time.[1 2] About 10% of patients eventually become wheelchair bound[3] and up to two-thirds of patients have disease related problems with their daily activities.[4]

FSHD shows an autosomal inheritance pattern with a high penetrance (95% at the age of 20) and has a prevalence of 1 in 20 000.[1 3] However, this latter figure is certainly an underestimate because of the recently estimated high new mutation frequency of 9·6%, corresponding with one new case per 320 000 live births.[5] In the majority of FSHD families the gene defect (FSHD1) has been localised to chromosome 4q35,[6–8] although a number of classical FSHD families have been reported that exclude this region,[9] indicating locus heterogeneity in at least 5% of FSHD families.

Recently, specific deletions of chromosome 4q35 have been detected using probe p13E-11 (D4F104S1), which play a causal role in the aetiology of FSHD1.[10–15] These deletions occur in highly polymorphic *Eco*RI fragments which generally vary in length from 50 kb to 300 kb, as was shown on pulsed field gel electrophoresis (PFGE), and have a VNTR-like structure with a 3·3 kb repeat unit.[16] Owing to the deletion of an integral number of these 3·3 kb repeat units, as was assessed by both PFGE and "normal" gel electrophoresis, the *Eco*RI fragments usually become shorter than 35 kb in FSHD1 patients.[17] However, in the DNA of control subjects a reduced size of the p13E-11 *Eco*RI fragment was sometimes found. Recently the probe p13E-11 has shown that fragments with a similar 3·3 kb repeat polymorphism occur on chromosome 10qter.[18] In eight CEPH pedigrees, linkage of "normal" but <35 kb p13E-11 fragments was shown with markers D10S590, D10S180, and D10S212. Unfortunately, about 10% of the 10q linked polymorphic fragments are smaller than 35 kb, a size comparable to the 4q deletion fragments observed in FSHD1 patients.[18]

In this report we discuss the diagnostic protocol we currently apply for postnatal tests in cases of familial and sporadic FSHD1. Until the FSHD1 gene has been isolated, diagnosis will be dependent on indirect methods and will therefore be limited to either chromosome 4q35 linked families or to isolated patients who exhibit a new p13E-11 deletion mutation. Furthermore, the P13E-11 deletion fragments (<35 kb) are useful as a tool in the differential diagnosis of isolated muscular dystrophy patients. In addition, our first prenatal diagnosis will be described.

Methods

Families for pre- and postnatal testing are usually referred through one of the seven Clinical Genetics Centres in The Netherlands. Most of the sporadic patients are referred by neurologists after these patients have been diagnosed clinically as possibly having a muscular dystrophy, where FSHD has not yet been excluded. In these cases, DNA testing will serve as a tool in the exclusion of FSHD.

Blood is collected from all family members or, in the case of new patients, from both parents and the index patient. DNA is isolated by use of standard methods[19] which in our hands routinely yields high molecular weight DNA (>300 kb). In the case of prenatal testing, a chorionic villus sample is taken at 11 weeks of gestation. DNA from the chorionic villi is isolated by using the phenol extraction method as described previously.[20]

Typing for the DNA markers pH30 (D4S139), LILA5 (D4S163), and p13E-11 (D4F104S1) is performed as follows. DNA is digested by the appropriate restriction enzyme under conditions specified by the manufacturer (Pharmacia). After electrophoresis through a 0·5% to 0·7% agarose gel for 18 hours (36 hours for the P13E-11 *Eco*RI digests), the DNA is transferred to a Hybond N+(Amersham) membrane. Hybridisation is performed overnight at 65°C according to the method of Church and Gilbert.[21] Filters are washed to a stringency of 0·3 × SSC/0·1% SDS, followed by autoradiography using Kodak XAR film with an intensifying screen. For typing of the chromosome 10q STR markers D10S590 and D10S212, PCR amplification and detection is performed as described previously.[18]

Both pH30 (D4S139)[22] and LILA5 (D4S163)[23] detect, by Southern analysis with *Taq*I and *Pst*I respectively, a VNTR polymorphism. P13E-11 (D4F104S1)[11] identifies an *Eco*RI polymorphism; however, only a subset of the fragments will be detected using conventional agarose gels.[16]

DIAGNOSTIC PROTOCOL

Strategy in familial situations

To test for linkage to 4q35, DNA samples from the family members are simultaneously analysed by Southern blotting using *Eco*RI, *Pst*I, and *Taq*I digested DNA and hybridised with p13E-11, pH30, and LILA5, respectively. Evidence for FSHD1 linkage to 4q35 requires a lod score of at least +1·2, as discussed by Lunt *et al.*[3] Small families with a limited number of informative meioses (lod score <1·2) need to show complete segregation between FSHD1 and the 4q35 markers including p13E-11, and, preferably, exhibit recombination events between p13E-11 and the 10q loci D10S590 or D10S212 or both.

Only the presence of a p13E-11 *Eco*RI fragment smaller than 35 kb, which segregates with FSHD1 and originates from 4q35, can be further used for presymptomatic, post-symptomatic, or prenatal diagnosis.

Strategy in isolated cases

DNA from both the healthy parents and the index patient is required for analysis using p13E-11. If a p13E-11 *Eco*RI fragment smaller than 35 kb is detected, which is not present in either of the parents, the diagnosis FSHD1 is as good as confirmed assuming that the 10q locus does not generate de novo p13E-11 fragments associated with FSHD. In all these cases, non-paternity will be excluded by use of multiple, highly polymorphic markers or DNA fingerprinting. Haplotype analysis of additional family members might show the parental origin of the de novo mutation.

If one of the parents shares a <35 kb *Eco*RI p13E-11 fragment with the index patient, more family members will be tested and linkage studies using both chromosome 4q35 and 10qter markers will be carried out to determine the chromosomal origin of this <35 kb p13E-11 fragment.

For all cases tested, especially sporadic cases and those in which a de novo fragment with a suspicion of mosaicism was detected, grandparents (if available and willing to cooperate) were requested to give blood to determine the paternal origin of the mutated chromosome and prove the de novo mutation. If no DNA from the parents is available for analysis a <35 kb P13E-11 band is indicative of FSHD1 (95%); in about 10% of controls a shortened P13E-11 *Eco*RI fragment (chromosome 10q) is present.[19] If no shortened P13E-11 band is detected, FSHD1 becomes less likely, because 5% genetic heterogeneity is anticipated.

Results

By lengthy electrophoresis (36 hours) good resolution of the high molecular weight *Eco*RI fragments (30 to 35 kb) is achieved. This higher resolving power was exploited in the analysis

Molecular genetic data for FSHD

	No	*p13E-11 size (kb)*	*No*	*Linkage to p13E-11*		*No*	*Parental origin*	*Probability of FSHD1 (%)*
Familial (index cases)	33	<35	27	4q		4		99*
				Non 10q		8		99*
				De novo		3	1 GM 2 GP†	100
				4q‡		2		95
				10q		2		10
				4q or 10q		8		90
		>35	6	?		6§		<10
Sporadic (index cases)	57	<35	25	De novo	16	5 M 2 P‖		100
				4q or 10q		9		90
		>35	32					<10

* All known "short" p13E-11 chromosome 4 bands are FSHD associated.
† 1 mosaic.
‡ In these cases there is a <35 kb p13E-11 band present which does not segregate with the FSHD or the chromosome 4 haplotype (see fig 1).
§ Families too small for linkage.
‖ 2 mosaics.

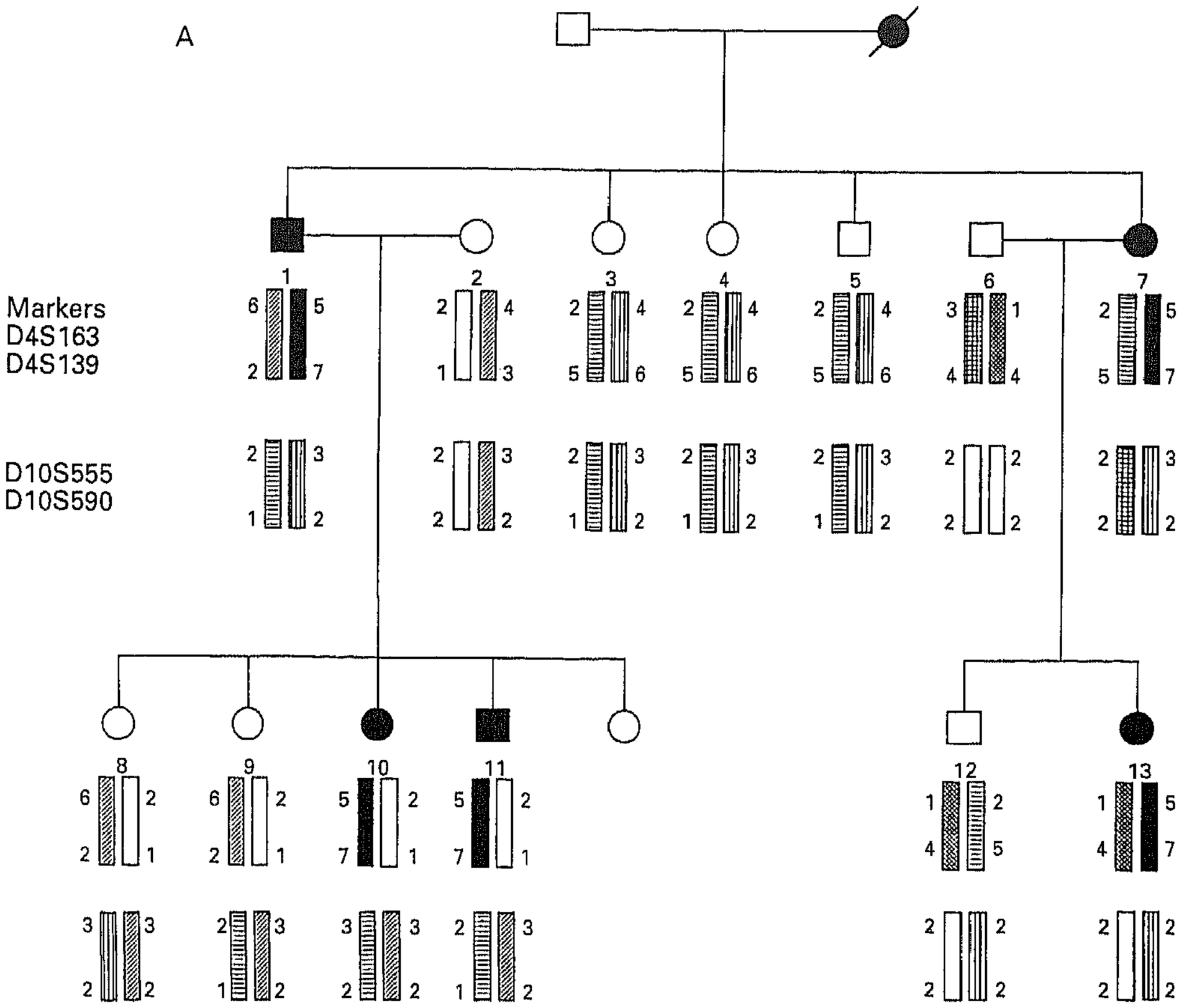

Figure 1 FSHD family 87. (A) In this pedigree FSHD (clinically confirmed by GWP) segregates with chromosome 4q35 markers D4S163 and D4S139. All affected subjects share the haplotype 5,7. Haplotypes for the chromosome 10q markers D10S555 and D10S590 are also depicted in the pedigree. (B) Southern blot results of EcoRI digested genomic DNA hybridised with the probe P13E-11 also shows a shortened fragment of 27 kb in the DNA of unaffected subjects. This P13E-11 band does not segregate with either FSHD or the chromosome 4q haplotype nor with a chromosome 10q haplotype.

of patient material, rather than PFG electrophoresis which is not operational in our diagnostic setting.

In total, 33 families and 57 sporadic cases were referred for DNA analysis for exclusion of FSHD. A summary of the data is given in the table. All families were tested for linkage to 4q35 with the three probes p13E11 (D4F104S1), pH30 (D4S139), and LILA5 (D4S163).

In 27 of the 33 families, a p13E-11 *Eco*RI fragment smaller than 35 kb was observed in

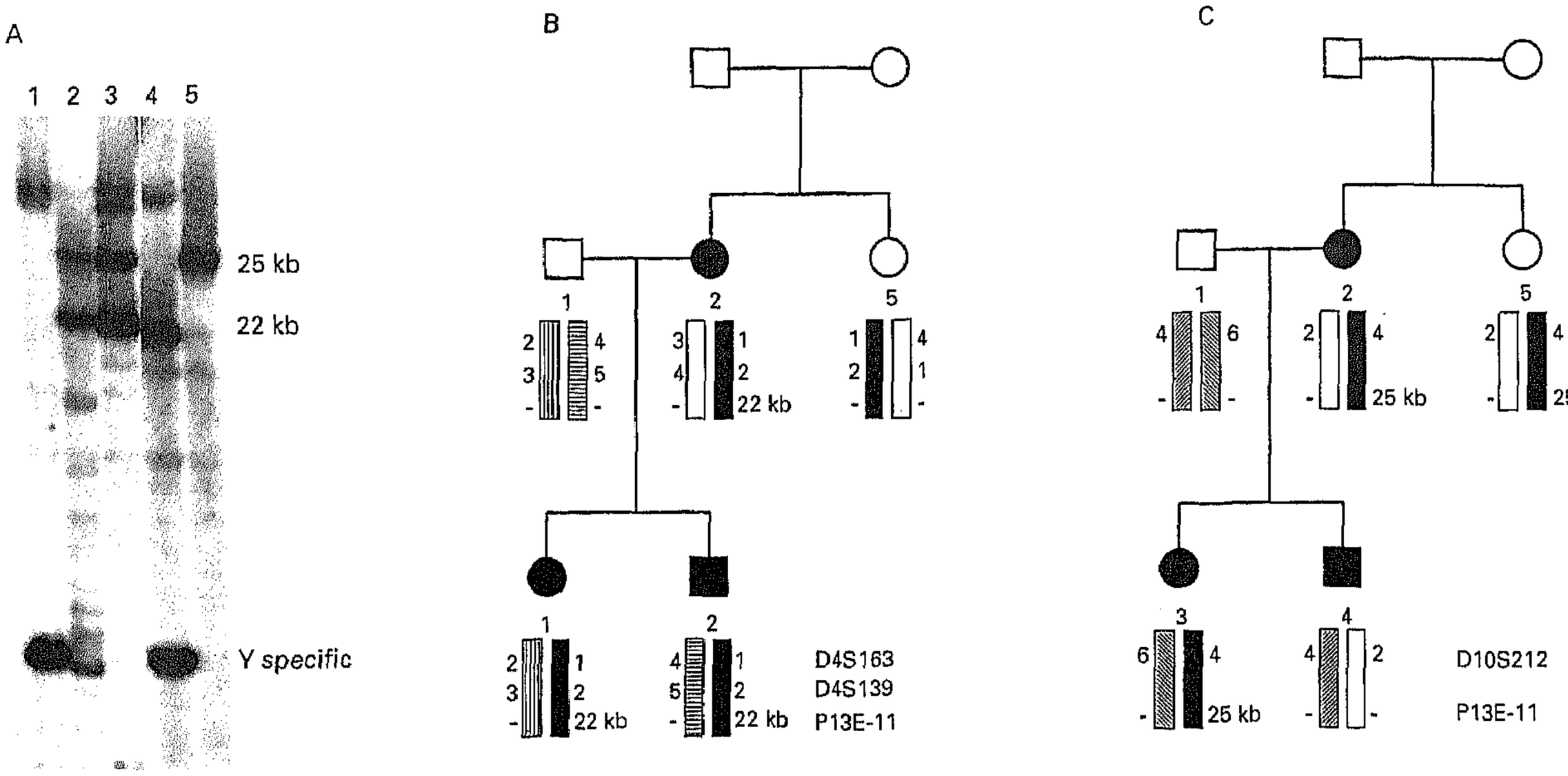

Figure 2 FSHD family 38. (A) In relatively small families, the investigation of both chromosome 4 and chromosome 10 markers can be extremely useful for correct interpretation of the p13E-11 data. In family fsh38, two p13E-11 fragments <35 kb (one of 22 kb and one of 25 kb) are detected by conventional Southern analysis. (B) Further DNA analysis suggests that the haplotype 2-1-22, (D4S163, D4S139, D4F104S1) segregates with the disease locus (FSHD1). However, subject 5, who does not show any symptoms of FSHD on clinical examination, shares this haplotype except for the 22 kb p13E-11 fragment. This inconsistency can be explained as follows. (1) The 22 kb fragment is associated with FSHD and subject 5 is not affected either because of a recombination or the fact that subject 2 is a new mutant. (2) Subject 5 is an asymptomatic carrier and FSHD is linked to 4q35 and the 22 kb fragment originates from chromosome 10 and is not associated with FSHD. (C) Chromosome 10 markers show that the 25 kb p13E-11 fragment segregates with chromosome 10q while the 22 kb fragment does not and therefore proves that it indeed segregates with 4q35 and thus is associated with FSHD1 in this family.

the DNA of the index patient; the size of the fragments ranged from 11 to 34 kb. The <35 kb p13E-11 fragment was shown to be linked (lod $> +1{\cdot}2$) to the FSHD1 gene in only four families. In eight additional families this <35 kb p13E-11 fragment most likely originated from 4q35 since it was excluded from 10qter by linkage analysis. In three families, the proband carried a de novo mutation. In eight small families the origin of the <35 kb p13E-11 fragment could not be determined. Two families showed a <35 kb allele which did not segregate with either FSHD or with 4q markers; therefore we assume that in these two families a >35 kb p13E-11 fragment might be associated with the disease. Fig 1 shows one of these two pedigrees haplotyped for both 4q35 and 10q; FSHD is linked to 4q35 but no clear cosegregation is observed with a <35 kb P13E-11 allele. In six familial cases, suggestive of FSHD, no <35 kb allele was detected; these families were too small to include or exclude 4q or 10q involvement. In total, 17 of the 33 "familial cases" (51%) satisfy either of the two following criteria. (1) The FSHD phenotype is linked to markers from chromosome 4q35 with a lod score of at least $+1{\cdot}2$, and (2) within the family a p13E-11 *Eco*RI fragment smaller than 35 kb segregates with FSHD1 and does not originate from chromosome 10qter; this is required to make DNA analysis in FSHD1 reliable enough for diagnostic purposes.

In 25 out of the 57 sporadic cases p13E-11 fragments were detected between 11 kb and 35 kb. Sixteen of these 25 cases were proven to be de novo mutations after analysing both parents. In five cases, neither or only one parent was available for analysis, and in four cases the index patient was found to share a small fragment with one of the parents. In the latter cases no additional family members were available for haplotype analysis using additional 4q35 and 10qter markers, leaving these cases inconclusive. The clinical information on most of the 32 patients, in whose DNA no <35 kb allele can be detected, is usually limited to "suggestive FSHD" in patients with limb-girdle-like symptoms with some facial weakness. Usually those patients are sent in by regional neurologists for refinement of the diagnosis.

In total, three cases of somatic mosaicism were detected among 19 de novo deletion mutants at the p13E-11 locus.

DIAGNOSTIC USE OF 10q MARKERS

In family fsh38, two p13E-11 alleles smaller than 28 kb were identified by Southern blot analysis using *Eco*RI (22 kb and 25 kb). To distinguish between the two possibilities (fig 2B), chromosome 10qter markers (fig 2C) were used to exclude that this 22 kb fragment originates from chromosome 10, and, therefore, that it must segregate with 4q35 and is associated with FSHD1 in this family.

SOMATIC MOSAICISM

In family fsh25, a de novo 16·5 kb p13E-11 fragment was detected in the index patient (subject 3, fig 3). Despite the fact that equal amounts of DNA were loaded in every lane, the clinically healthy father presented the same

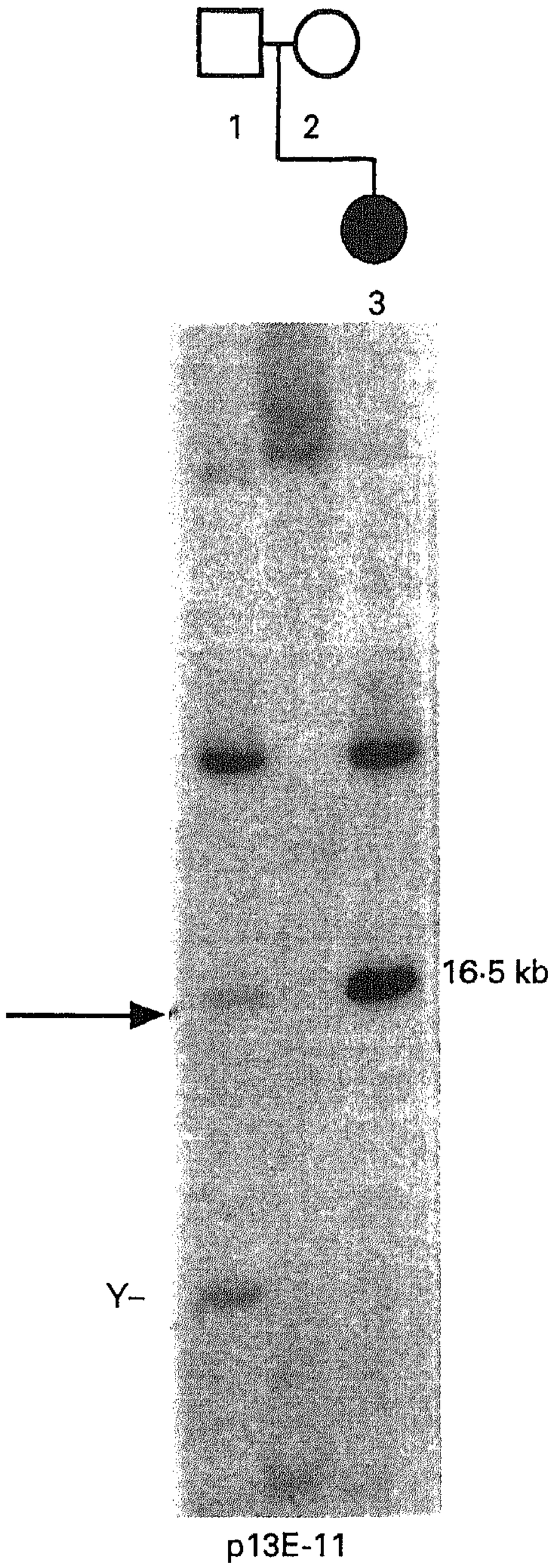

Figure 3 Somatic mosaic. FSHD family 25. A de novo 16·5 kb P13E-11 fragment was detected in a sporadic FSHD patient. Upon critical inspection and repeated analysis a weak signal of the 16·5 kb band was present in the DNA of the father (indicated by an arrow), highlighting somatic mosaicism for this de novo mutation.

16·5 kb fragment, except that in his case the fragment had a much weaker intensity (fig 3, indicated by an arrow). The signal remained after repeated analysis, highlighting the mosaic status of subject 1 for this de novo mutation.

PRENATAL DIAGNOSIS

So far, only one couple has requested prenatal diagnosis. The index patient in family fsh22 (fig 4, subject 3) was recently diagnosed as a sporadic FSHD1 patient by showing a de novo 22 kb p13E-11 fragment (fig 4). Non-paternity was excluded by analysing different random microsatellite markers and the two highly polymorphic VNTR markers pH30 (D4S139) and LILA5 (D4S163). After his spouse became pregnant they were referred to determine the FSHD1 status of the fetus. As shown in lane 8 the fetus inherited the rearranged 22 kb fragment from the father (subject 3) that is associated with FSHD1. The couple decided to terminate this pregnancy. Haplotype analysis of the fetus showed that the deletion leading to this de novo 22 kb fragment occurred on the grandmaternal chromosome 4 (subject 1).

Discussion

The use of indirect methods for diagnostic purposes in FSHD1 has been hampered since its localisation in 1990 because (1) no flanking markers are available, (2) genetic heterogeneity exists in about 5 to 10% of the families, and (3) homologous chromosome 10q alleles identified by p13E-11 occur, whose size interferes with the FSHD associated deletion fragments, making interpretation of data very difficult.

In this report we describe the diagnostic protocol we have developed for pre- or postnatal testing in those FSHD families that meet the following two criteria: (1) the FSHD phenotype is linked to markers from chromosome 4q35 with a lod score of at least +1·2, and (2) within this family a p13E-11 *Eco*RI fragment smaller than 35 kb segregates with FSHD1, and does not originate from chromosome 10qter. A <35 kb *Eco*RI p13E-11 fragment which originates from 4q35 is diagnostic for FSHD1 with a probability of over 99% because all known fragments <35 kb and linked to chromosomes 4q35 are associated with FSHD.

From the 33 FSHD families that were referred to us for DNA analysis, 81% (that is, 27 families) showed a <35 kb p13E-11 fragment. From these 25 families only 15 (55%) fulfil the criteria (sufficient proof of linkage or a de novo deletion of P13E-11) for either presymptomatic or prenatal DNA testing. In two of the 33 families a <35 kb P13E-11 allele was detected but was not diagnostic because it did not segregate with either the FSHD or the 4q35 markers (fig 1). In these two families presymptomatic or prenatal DNA testing is only possible using linkage studies. In large 4q35 linked FSHD families that lack a diagnostic p13E-11 fragment (estimated to occur in about 5% of families), the reliability of detecting carrier status decreases to 85%, based on a 5% probability of recombination between D4S139 and FSHD1. An alternative in these families would be to analyse all p13E-11 fragments using pulsed field gel electrophoresis (PFGE).[17] However, PFGE is too labour intensive and practically difficult to perform as a standard diagnostic test.

In the case of sporadic patients identification of a de novo deletion confirms the diagnosis of FSHD1. The failure to detect a new fragment in patients who are suspected of having FSHD

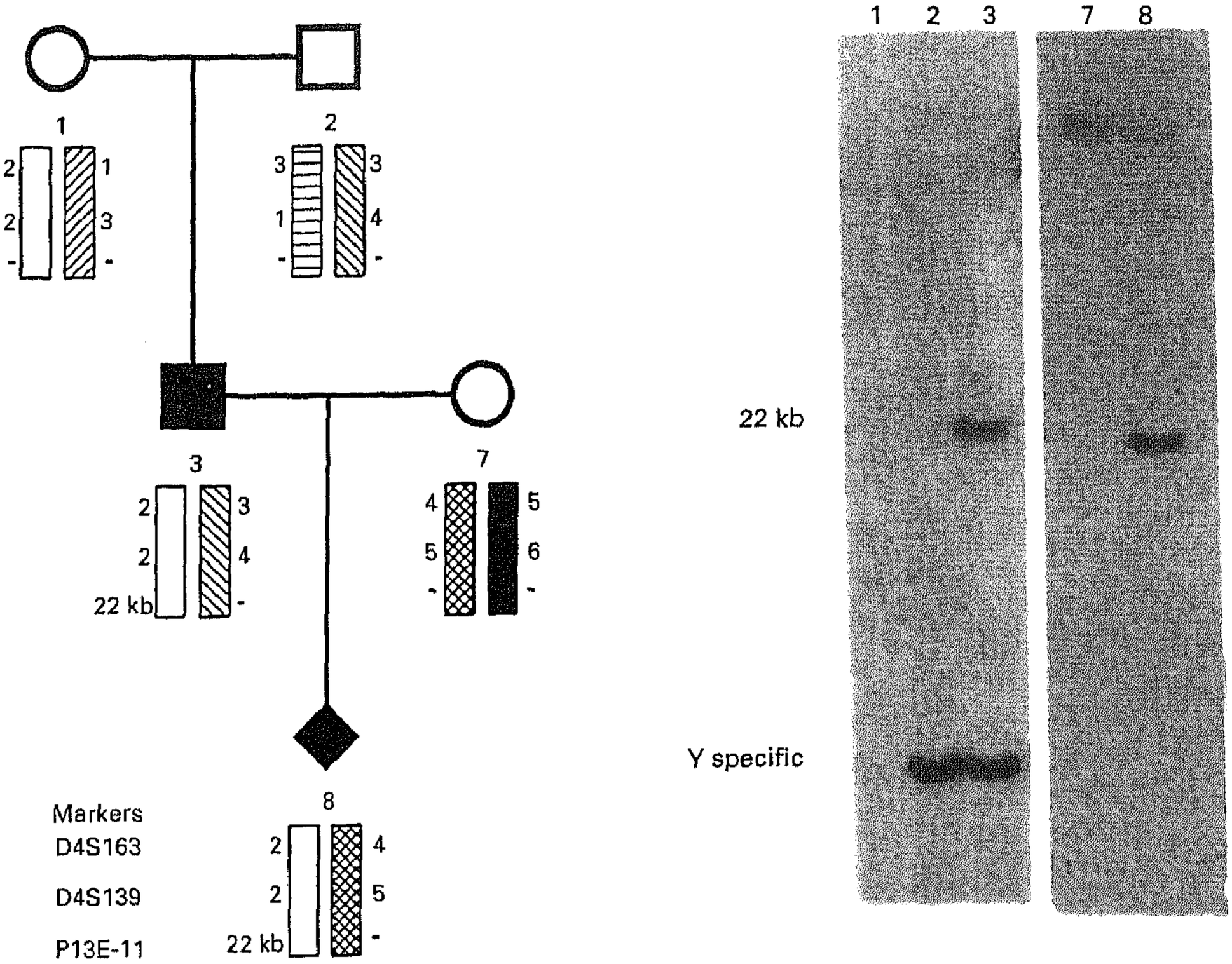

Figure 4 Prenatal diagnosis. FSHD family 22. Subject 3, diagnosed as a sporadic FSHD patient, requested prenatal diagnosis by DNA analysis. In his DNA a 22 kb p13E-11 EcoRI fragment was detected which was not present in the DNA of his parents (subjects 1 and 2). Non-paternity was excluded by analysing different random microsatellite markers and the two highly polymorphic VNTR markers D4S139 and D4S163. Also the DNA of his wife was tested to exclude the presence of an interfering "short" p13E-11 allele. In the 11th week of gestation chorionic villi were taken and analysed. As shown in lane 8 the fetus inherited the rearranged 22 kb fragment of the father (subject 3) associated with FSHD. The couple decided to terminate this pregnancy. The deletion leading to the de novo 22 kb fragment occurred on one of the chromosomes transmitted by the grandmother (subject 1).

makes it very unlikely (<10%) that these patients suffer from FSHD1. Owing to the presence of an undetectable mutation, associated with FSHD1, or to locus heterogeneity (each anticipated to occur in <5%), these patients are most likely (>90%) to be clinically misdiagnosed.

Our first prenatal diagnosis was carried out in a well defined family situation where a de novo mutation could easily be followed in a subsequent generation (fig 4).

The observation of three cases of presumed somatic and germline mosaicism in a total of 19 de novo mutations (table) has consequences for the recurrence risk in proven de novo mutation cases. By analogy with earlier empirical data on recurrence risk owing to germinal mosaicism for Duchenne muscular dystrophy mutations (DMD),[24] for FSHD1 we would propose about a 10% risk for a sib of a de novo case being a carrier. The figure of 10% (10–15%) is based on the observation that, as in DMD, both somatic and germinal mosaics are detected, indicating that both types of mutations occur early in embryogenesis and therefore will give rise to a high percentage of mutated germ cells.

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